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Induction and characterization of a replication competent cervid endogenous gammaretrovirus (CrERV) from mule deer cells

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ABSTRACT

Endogenous retroviruses (ERVs) were acquired during evolution of their host organisms after infection and mendelian inheritance in the germline by their exogenous counterparts. The ERVs can spread in the host genome and in some cases they affect the host phenotype. The cervid endogenous gammaretrovirus (CrERV) is one of only a few well-defined examples of evolutionarily recent invasion of mammalian genome by retroviruses. Thousands of insertionally polymorphic CrERV integration sites have been detected in wild ranging mule deer (*Odocoileus hemionus*) host populations. Here, we describe for the first time induction of replication competent CrERV by cocultivation of deer and human cells. We characterize the physical properties and tropism of the induced virus. The genomic sequence of the induced virus is phylogenetically related to the evolutionarily young endogenous CrERVs described so far. We also describe the level of replication block of CrERV on deer cells and its capacity to establish superinfection interference.

Introduction

Endogenous retrovirus sequences constitute an integral part of all vertebrate genomes. They are generated following infection of the germline lineage of the host by an exogenous retrovirus and subsequent vertical inheritance of the integrated provirus form (Feschotte and Gilbert, 2012). The ERVs are classified into a large number of groups, whose diversity exceeds the currently circulating retrovirus species (Blomberg et al., 2009; Hayward et al., 2015). After the initial establishment of an integrated virus copy, which serves as a founder for a specific ERV group, further amplification and creation of new copies is enabled either by reinfection or by intracellular retrotransposition in the germline (Dewannieux et al., 2004; Jern and Coffin, 2008; Kanda et al., 2013).

Uncontrolled proliferation of ERVs in the genome would cause a burden for the host through mutagenic and various other effects. Therefore, there are multiple mechanisms that keep ERV expression and replication under control, most notably by transcriptional silencing (Liu et al., 2014; Rowe and Trono, 2011; Turelli et al., 2014). On the other hand, ERVs can be utilized for protection of the host from infecting retroviruses, a concept dubbed as “fighting fire with fire” (Malfavon-Borja and Feschotte, 2015). There are several well-documented cases in chickens, mice, cats and sheep, where endogenous envelope (Env) proteins can prevent the cell surface receptors from interacting with incoming retrovirus, resulting in a block of cellular entry (Malfavon-Borja and Feschotte, 2015). ERV-encoded proteins can also cause inhibition at several post-entry stages of infection (Arnaud et al., 2007b; Best et al., 1996; Monde et al., 2012). Another important way how ERVs can influence the outcome of retroviral infection is through recombination. ERV genomes can recombine among different endogenous loci or with related exogenous retroviruses. This can lead to the generation of fully infectious virus from two defective ERV genomes, or to the altered properties, for example altered tropism, of the exogenous partner involved in the recombination (Anai et al., 2012; Levy, 2008; Paprotka et al., 2011; Shimode et al., 2015; Young et al., 2012). In addition, through recombination with cellular genes, ERVs can form acutely transforming retroviruses (Kozak, 2015).

A practical classification is to consider ERVs as either “ancient” or “modern”, based on the time when they infiltrated the host genome (Armezzani et al., 2014). Most ERVs belong to the ancient category, where the genome invasion occurred long time ago in the evolutionary history of the host species, usually before the last speciation. Consequently, the individual ERV integrations are fixed in the host population, or even shared in phylogenetically related species. Modern ERVs entered the host genome more recently, mostly after speciation. Such ERV integrations have typically not yet reached fixation or been lost from the host lineage. At that stage, they are present in some individuals and absent in others,

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which is denoted as insertional polymorphism. There are only a few well-studied examples of modern ERVs, these include the koala retrovirus (KoRV), endogenous Jaagsiekte sheep retroviruses (enJSRVs), porcine endogenous retroviruses (PERVs), endogenous feline leukemia viruses (enFeLVs) and other feline endogenous retroviruses (ERV-DCs), various mouse ERVs, and cervid endogenous gammaretrovirus (CrERV). The research on these viruses has lead to important insights into the process of genome invasion by an ERV and of the changes that accompany endogenization (Anai et al., 2012; Arnaud et al., 2007a; Lavillette and Kabat, 2004; Li et al., 2012; Oliveira et al., 2007; Tarlinton et al., 2006). In koalas, sheep and cats and mice, exogenous counterparts of the respective ERVs are circulating in natural populations and are associated with disease (Armezzani et al., 2014; Kozak, 2015; Levy, 2008; Xu et al., 2015). Replication-competent variants of PERV have also been reported (Preuss et al., 2006). In our previous studies we have advanced knowledge of the CrERV-mule deer model (Bao et al., 2014; Elleder et al., 2012; Kamath et al., 2014; Wittekindt et al., 2010). We have described an extensive collection of thousands of polymorphic endogenous retrovirus integration sites, comprehensively documenting the recent invasion of mule deer genomes by CrERV. The integration site patterns in individual deer were analyzed and revealed fine population structure and history of wild mule deer populations, with better resolution than in a parallel analysis performed with microsatellite markers (Kamath et al., 2014). However, all our previous work was focused on analysis of the integrated CrERV DNA or of the viral RNA expression. We have never obtained conclusive evidence of virus production or replication. Therefore, we attempted to replicate a previously published experiment (Aaronson et al., 1976), where primary blacktail deer (*O. hemionus colubmianus*, a subspecies of mule deer) cells were cocultured with human cell line. This led to the induction of replication competent gammaretrovirus species of hitherto unknown sequence, denoted deer kidney virus (DKV) (Aaronson et al., 1976; Barbacid et al., 1980).

In this study we report a successful induction of replication-competent CrERV from coculture of deer cells with a susceptible human cell line. We have characterized the physical properties of the induced virus, its phylogenetic relatedness to known endogenous CrERV copies, and its infectivity on deer and human cells. We also analyzed the capacity of the induced virus to establish interference to superinfection.

Results

CrERV can be induced by coculture of deer and human cells

Black-tailed deer primary kidney cells (OHK) and a human rhabdomyosarcoma cell line A673 were used in the coculture experiment. These cells were the same as those used in the original protocol (Aaronson et al., 1976). After approximately 30 days, RT activity in culture medium could be detected by a sensitive product-enhanced RT (PERT) assay (Fig. 1). At this point we stopped adding the OHK cells, which had served as a source of the induced virus. Due to much faster growth, only the human cells remained presumably in the subsequent continuation of the coculture. The RT activity continued to increase and eventually reached a plateau. The resulting RT level was still very low, approximately a thousand times lower than the values obtained for another endogenous gammaretrovirus, PERV (porcine endogenous retrovirus). The human and deer cells cultured separately tested negative in the PERT assay (data not shown). To confirm the identity of the induced virus species, viral cDNA was prepared from ultracentrifugation-concentrated culture fluids. Sequences highly identical to CrERV were obtained (full sequence of induced CrERV [CrERV-IND] is reported below). Therefore it is highly probable that DKV described by Aaronson, from which no sequence data is available, is identical to our recently reported CrERV.

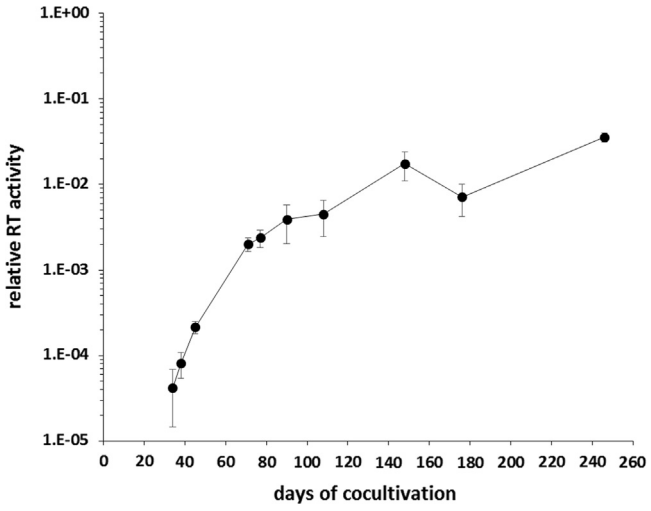

Fig. 1. Induction of virus from *O. hemionus* primary kidney cells cocultivated with human rhabdomyosarcoma cell line. At the indicated times, the RT activity in the culture supernatants was measured by a sensitive PERT assay. The results are expressed as means and standard deviations from triplicate assays.

CrERV particles

Next we examined whether the RT activity obtained from the coculture experiment belonged to particles of expected retrovirus buoyant density. The pelleted CrERV-IND was separated on iodixanol gradient and individual gradient fractions were tested by the PERT assay. For comparison we used virus particles of well-described endogenous gammaretrovirus, PERV (Bartosch et al., 2004). The RT activity peaked around the expected density of 1.1 g/ml, typical for retrovirus particles (Contreras-Galindo et al., 2012) (Fig 2A). We also obtained electron micrographs of both CrERV-IND and PERV (Fig. 2B).

The induced CrERV is infectious and xenotropic

Then, we evaluated the infectivity of the CrERV-IND particles. The virus inoculum from the coculture was used to infect naïve human and deer cells, and infectivity was assessed by the appearance of RT activity in the culture medium. Both human A673 and HEK 293 T cells could be infected (Fig. 4), however no RT activity was detected upon infection of deer OHK cells (data not shown). This is consistent with xenotropic characteristics of the induced virus, and is in agreement with observations reported by Aaronson et al. (1976) for DKV.

Relationship of induced CrERV sequence with known endogenous proviruses and construction of infectious molecular clone

To obtain the full sequence of CrERV-IND, we have amplified the provirus DNA from CrERV-infected HEK 293 T cells by long-range PCR. This sequence (deposited in Genbank under accession number KP261824) was 9,027 nucleotides long and had high identity across the entire length with a previously reported complete provirus genome, denoted CrERV-in7 (Elleder et al., 2012). There were intact open reading frames for all viral genes, *gag*, *pro/pol*, and *env* (Fig. 3B). We performed phylogenetic comparison of CrERV-IND with a set of previously published twelve endogenous CrERVs (Kamath et al., 2014). Because the full sequences of these CrERVs are not known, we have used an alignment of approximately 1.1 kb region in the 3'end of the virus genome to create the phylogeny. This region was identified

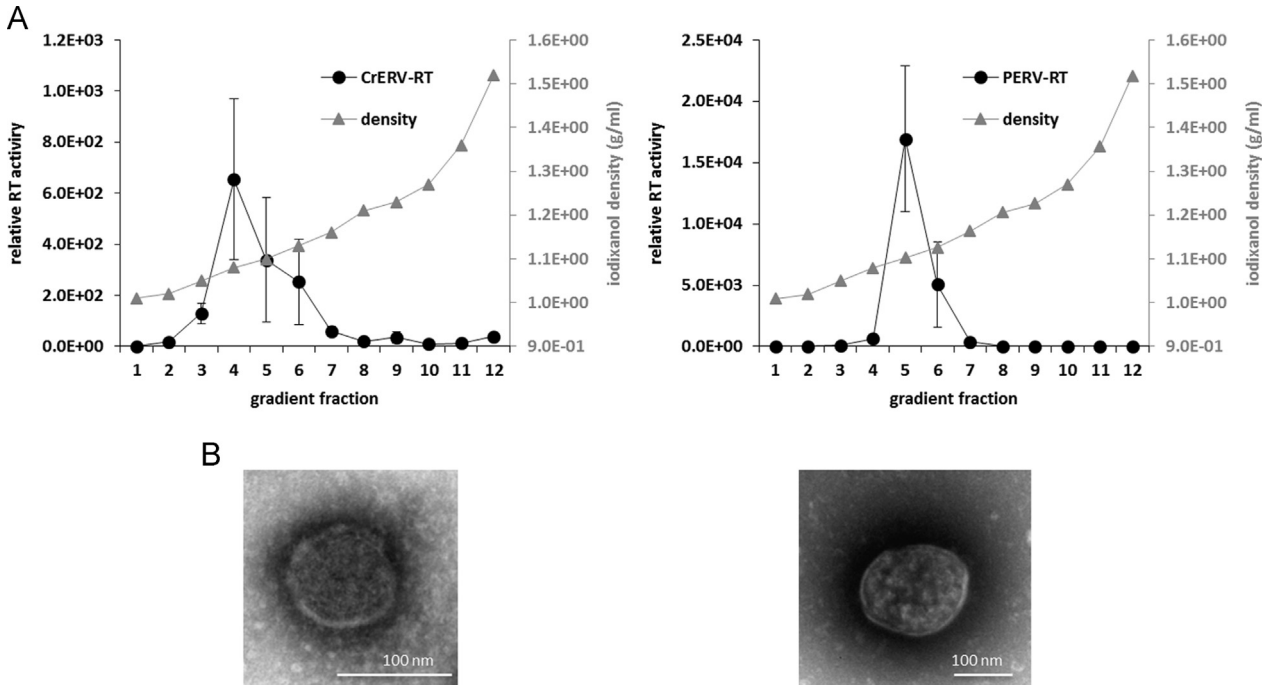

Fig. 2. Characterization of CrERV particles. (A) Pellets containing ultracentrifugation-concentrated virus particles were resuspended in PBS and layered on top of 10–50% stepwise iodixanol gradients. Following 17 h of centrifugation at 209,000 g, aliquots from each fraction were tested for RT activity by the PERT assay. Averages and standard deviations from triplicate assays are shown as black circles. Density of individual gradient fractions is depicted by gray triangles, with values displayed on the right axis. (B) Electron micrographs of retrovirus particles obtained by negative staining are shown, with 100 nm scale bar highlighted. In both A and B, CrERV is positioned on the left and PERV on the right.

previously (Kamath et al., 2014) to be convenient for phylogenetic analysis because it minimizes the influence of recombinant sequences. The CrERV-IND sequence clustered with good support with the large group of insertionally polymorphic, evolutionarily young CrERVs (Fig. 3 A). The CrERV-in1, which represents an evolutionarily old integration, was located on a separate branch.

We further compared the CrERV-IND sequence with four CrERVs for which we have full genomes available. Consistent with the results from the phylogenetic tree, the closest sequence to CrERV-IND was CrERV-in8, with only 24 genetic changes scattered over the entire 9- kb genome (Fig. 3B). CrERV-in4 and CrERV-in7 had 26 and 41 changes, respectively, relative to the CrERV-IND, and CrERV-in5 was even more distant. The ratio of nonsynonymous to synonymous substitutions in the coding regions was always higher in *env* than in *gag* and *pro/pol* (Fig. 3B). This is consistent with a higher degree of purifying selection in *gag* and *pro/pol* than in the *env* gene.

To standardize the work with the CrERV-IND, a molecular clone was constructed by subcloning the long range PCR product into pGEM-T Easy plasmid vector. The resulting construct pCrERV-IND was replication competent following transfection into HEK 293 T cells and subsequent infection of naïve cells. All subsequent analyses were performed with virus derived from this molecular clone.

CrERV infection kinetics in human cells

The RT activity of the CrERV-IND was very low even on permissive human cells. This is caused at least in part by very low titer of the virus. In end-point dilution experiments, the CrERV infectious titer on HEK 293 T cells was 10^{2-3} per ml. In addition, we tried to evaluate the infection kinetics using quantitative PCR methods. PERV was used again for comparison, and both viral inocula were normalized for RT activity. We have used both standard SYBR green real-time quantitative PCR and a highly sensitive digital droplet PCR method. The assay was detecting the newly formed virus DNA in the *env* gene region, corresponding to intermediate products of reverse transcription. The products could be detected several hours after infection of human cells and peaked around 8–24 h p.i., as described for other retroviruses (Mohammadi et al., 2013) (Fig. 4, left). The levels of newly made CrERV DNA were very low, about hundred times lower than for PERV. Based on the peak values being below $1x10^{-3}$ CrERV DNA copies per diploid cell genome, we estimate that less that 1 in 1000 cells was infected. However, during long-term culture of infected HEK 293 T cells, presumably all cells become infected in the course of about 3 weeks, because the virus DNA level reaches a plateau with values close to one copy per diploid cell genome (Fig. 4, right).

Early block of CrERV infection on deer cells, caused presumably by receptor interference

The induced CrERV was unable to productively infect deer cells and we wanted to characterize the level of the putative replication block. PCR-based assays used for human cells were not applicable because of the large background of hundreds of endogenous CrERV copies (Elleder et al., 2012) that would co-amplify together with the newly formed virus DNA in deer cells. Other relevant approaches, namely CrERV ENV pseudotypes of MLV and marker rescue assays were technically unsuccessful even after repeated attempts. Therefore, we used an alternative approach and by in vitro mutagenesis of the CrERV-IND molecular clone, we generated a sequence variant CrERV-mut. This construct contained several silent mutations in the *pol* gene (Fig. 5A). We have selected nucleotide variants not present in any of the previously described CrERV endogenous copies (Elleder et al., 2012; Kamath et al., 2014). This allowed us to design PCR primers that amplified only the newly generated CrERVmut DNA and not any of the endogenous CrERV copies or the parental CrERV virus (Fig. 5B, lanes 7 and 8). CrERV-mut virus was infectious, because it could infect naïve HEK-293 T cells (Fig. 5B, lanes 5 and 6). However, CrERV-mut newly made viral DNA was not detectable upon infection of deer cells (Fig. 5B, lanes 1 and 2). Therefore, either

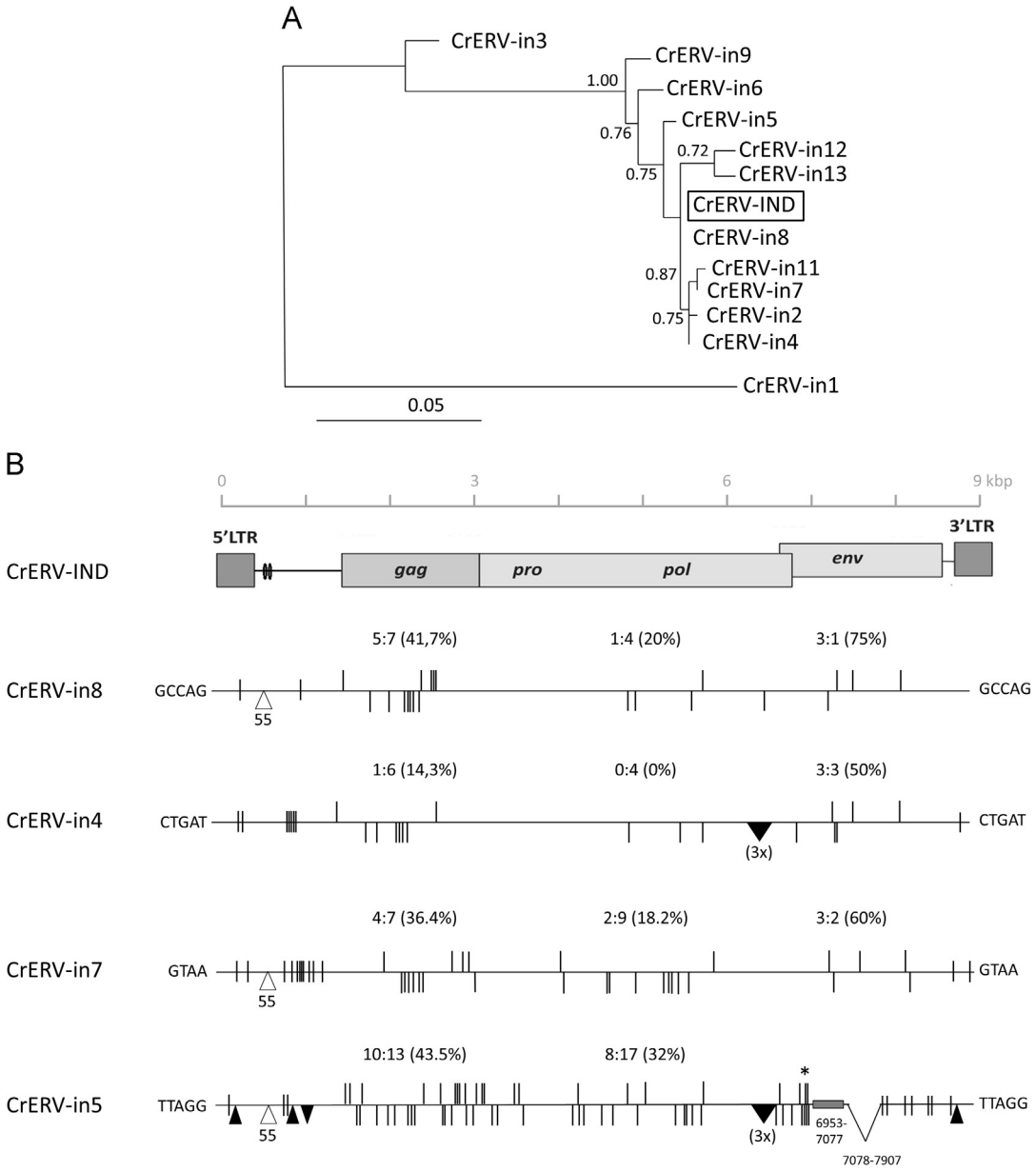

Fig. 3. Relationships between CrERV-IND sequence and endogenous CrERVs. (A) Phylogenetic tree generated from alignment of 1.1 kb region in the 3′end of the CrERV-IND and endogenous CrERVs. CrERV-IND position is highlighted by an open box. Bootstrap supports higher than 0.7 are indicated at the tree nodes. The branch length is proportional to the number of substitutions per site, with scale bar shown below the tree. (B) Comparison of the CrERV-IND sequence with four full endogenous CrERV genomes. The CrERV-IND genome is shown as a schematic at the top. Virus genes and LTRs are shown as shaded boxes and two direct 55- bp repeats in the leader region are displayed as small ovals. Every genome is depicted by horizontal black line, flanked with short target site duplication (TSD) sequences that arise during retrovirus integration. Short vertical lines indicate single nucleotide mismatches relative to the CrERV-IND sequence. Mismatches inside the coding regions for *gag*, *pro/pol* and *env* are shown above and below the horizontal line for the changes that result in nonsynonymous and synonymous substitutions, respectively, in the corresponding virus ORF. The numbers above each line represent the number of nonsynonymous: number of synonymous substitutions in each gene. The percentage of nonsynonymous mutations out of total mutations is shown in parentheses. Upward pointing black triangles indicate single nucleotide insertions, downward pointing triangles are single nucleotide deletions (the sign 3x denotes three closely spaced deletions). Open triangles represent insertion of third 55- bp direct repeat in the leader region. Premature stop codons are indicated by asterisk. Gray shaded box shows a region of CrERV-in5 *env* gene that is unalignable with CrERV-IND *env*. V-shaped line indicate region with large deletion.

receptor-mediated entry or some of the earliest steps of CrERV replication (virus uncoating, RT initiation), that precede virus DNA synthesis, are deficient in deer cells. To further test if receptor interference can block CrERV infection, we used the human cells chronically infected with CrERV. These cells presumably all harbor integrated CrERV (Fig. 4B) and express the virus envelope and therefore have the capacity to block cellular receptors used for virus entry. The chronically infected cells did not support generation of CrERVmut virus DNA (Fig. 5B, lanes 3 and 4) This is consistent with receptor interference being the cause of the resistance to CrERV on both deer cells and chronically infected human cells.

Discussion

Here we report the induction and characterization of a replication competent endogenous gammaretrovirus, CrERV, from mule deer cells in a coculture experiment. The process that led to the induction of CrERV production from the coculture is not known. It could have involved low production of infectious virus particles from deer cells, which then infected the permissive human cells. We have not detected virus production from deer cells by the PERT assay. However, this production could have been extremely low or intermittent. Among other potential mechanisms are rare spontaneous cell fusion

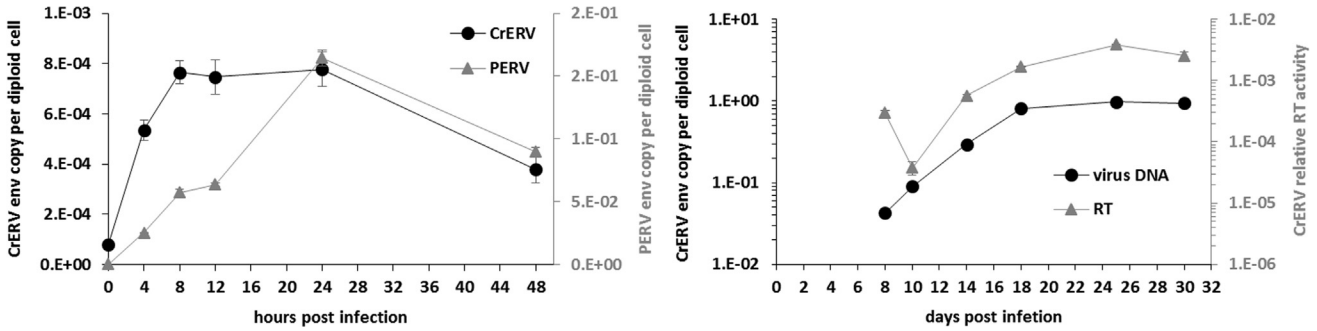

Fig. 4. CrERV infection kinetics on HEK 293 T cells. (Left) Quantification of newly made virus DNA in the first two days post infection. At indicated times, infected cells were collected and cellular lysates were prepared as described in Methods. Real-time PCR assays using MESA green and primers complementary to the virus *env* were used to quantify virus DNA levels. CrERV is depicted by black circles and PERV by gray triangles. (Right) Long-term infection of HEK 293 T cells by CrERV. Virus DNA levels in cellular lysates were quantified by digital droplet PCR method, using the same *env*-based primers as in the experiment depicted on the left diagram. The resulting values are shown as black circles. RT activity was determined from culture supernatants in parallel and is shown as gray triangles. The infection experiments were performed at least twice with similar results. Values from one representative experiment are shown, with averages and standard deviations from quantitative assays performed in triplicate.

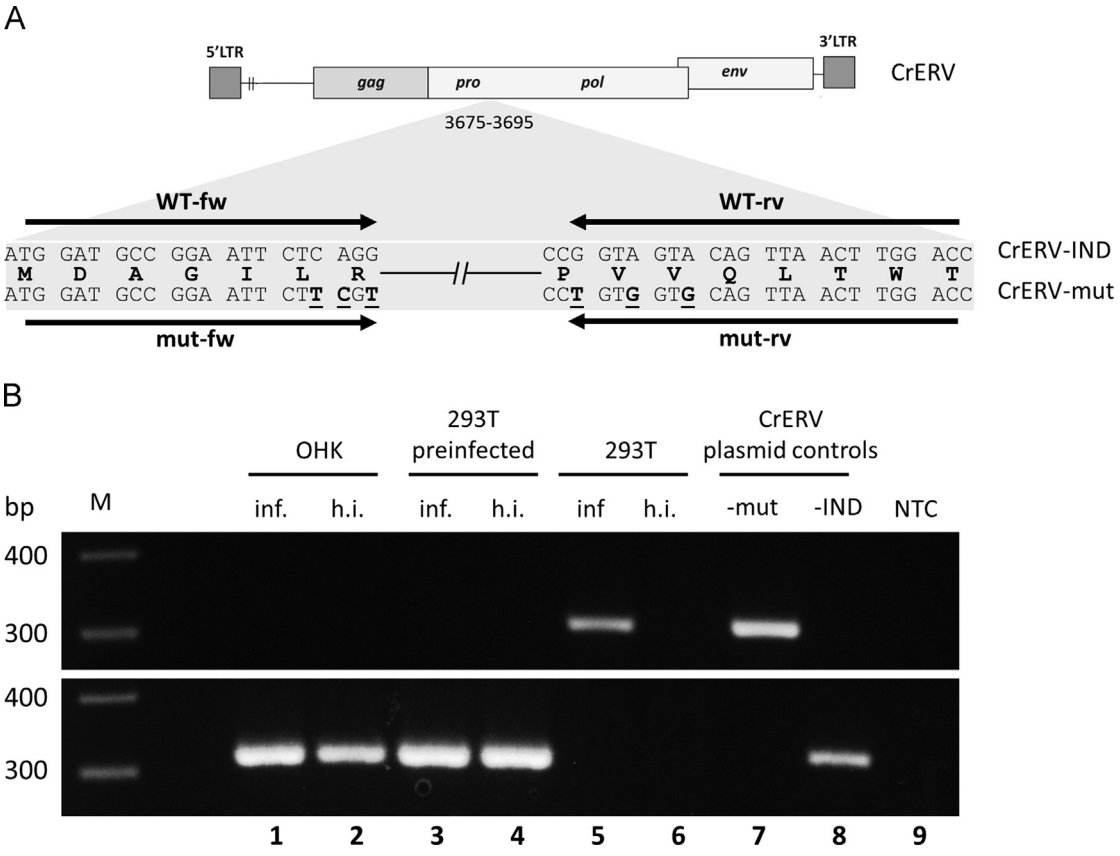

Fig. 5. Infection of human and deer cells by a CrERV sequence variant generated by in vitro mutagenesis, CrERV-mut. (A) A schematic depicting position and sequence of the primer pairs that specifically detect only CrERV-IND or CrERV-mut DNA. The upper and lower sequences represent the CrERV-IND and CrERV-mut genome, respectively. The nucleotide substitutions generated in CrERV-mut are underlined and bold. The arrows indicate positions of primers. Primers WT-fw and WT-rv amplify only the CrERV-IND virus DNA. Primers mut-fw and mut-rv amplify specifically the CrERV-mut DNA. (B) CrERV-mut was used to infect deer OHK cells (lane 1), HEK 293 T cells (lane 5) and HEK 293 T cells chronically infected with CrERV-IND (lane 3). Heat-inactivated (h.i.) virus was used in each case as a negative control to exclude virus DNA contamination (lanes 2, 4, and 6). Cells were harvested 20 h after infection and cellular lysates were prepared as described in Methods. CrERV-mut (lane 7) and CrERV-IND (lane 8) plasmid DNA was used as a control for specificity of PCR amplification. The upper panel shows PCR products generated with primers mut-fw and mut-rv, which detect specifically the CrERV-mut DNA. The lower panel shows PCR products generated with primers WT-fw and WT-rv. These primers amplify the "wild-type" variants of CrERV, i.e. the endogenous CrERVs in deer cells (lanes 1 and 2), and CrERV-IND in chronically infected 293 T cells (lanes 3 and 4). The experiments were performed twice with identical results; one representative experiment is displayed. M, molecular size marker; NTC, non-template control.

events, direct cell-to-cell transmission through virological synapses, or even transfer via exosomes (Sattentau, 2010; Wurdinger et al., 2012). In general, virus transmission or rescue by cocultivation was proven to be the most effective method for both endogenous and exogenous retroviruses (Agosto et al., 2015; Patience et al., 1997; Svoboda et al., 1963; Xu et al., 2013).

Several lines of evidence show that the induced CrERV is identical to the previously reported DKV, although the DKV genome has never been sequenced by the Aaronson laboratory. First, we tried to reproduce the setting of the original experiment, most importantly we used the same cell types in the coculture. Second, immunological methods established that DKV belonged to gammaretroviruses (Aaronson et al., 1976; Barbacid et al., 1980), as does CrERV. Third, DNA hybridization techniques using DKV probes established the presence of closely related endogenous viruses in all tested cervid species (Tronick et al., 1977). More

distantly related endogenous retroviruses were detected in other artiodactyls, e.g. in sheep. This pattern of distribution is consistent with the detection of various CrERV-related sequences in mammalian genomes (Elleder et al., 2012). A last argument pointing to the identity between CrERV and DKV is the similar tropism of both viruses, discussed below. DKV was shown to replicate on human cells but not deer cells, same as CrERV. In addition, DKV had a narrow xenotropic host range; it could replicate on horse cells, but not in cells of several additional mammalian species (Aaronson et al., 1976).

The genomic sequence of CrERV-IND did not show any apparent defect; it had complete open reading frames for all viral genes. In addition, the construction of an infectious molecular clone excluded the theoretical possibility that a helper virus from deer cells was needed for the replication of CrERV-IND. The obtained sequence was closest to the group of evolutionarily young and highly insertionally polymorphic CrERVs. This is consistent with the assumption that the youngest ERVs retain the highest capacity to be mobilized to form infectious progeny. Indeed, the closest relative to CrERV-IND was CrERV-in8, which was extremely rare in the population study, with only 1 positive animal out of 262 total (Kamath et al., 2014). However, none of the endogenous CrERVs was completely identical to CrERV-IND, therefore we cannot determine the source element that gave rise to the induced virus. There are hundreds of endogenous CrERVs in each deer genome; moreover, the deer cells used in the coculture were different from those we analyzed previously. We cannot exclude that recombination events between several endogenous CrERVs were involved in the generation of CrERV-IND, similar scenario was described for several other ERVs (Anai et al., 2012; Levy, 2008; Shimode et al., 2015; Young et al., 2012). Interestingly, although we analyzed only a few CrERV genomes, there seems to be a trend towards higher degree of purifying selection in *gag* and *pro/pol* than in env. Loss of *env* has been identified as a factor determining greater expansion of ERVs within the genome (Magiorkinis et al., 2012).

The buoyant density of CrERV-IND and electron micrographs of virus particles did not show obvious aberrations. However, the infectivity of CrERV-IND was very low even on permissive human cells. This could be due either to inherent defect of the CrERV-IND genome or to low permissivity of the human cells. The early kinetics of virus DNA production seemed normal and the virus eventually infected presumably all cells in the culture.

To explain the xenotropic nature of CrERV-IND, we tried to determine the level of the replication block on deer cells. Using the genetically modified variant CrERV-mut, we have shown that the block occurred before the viral DNA synthesis. The most plausible explanation is the receptor interference caused by the expression of endogenous *env* genes. To support this explanation, we have further shown that CrERV has the capacity to establish superinfection resistance on chronically infected human cells. However, because the cellular receptor for CrERV is not known, we cannot exclude that it is mutated in deer. Selection for such mutations in receptors for ERVs has been documented in endogenous avian leukosis viruses (ALV) and murine leukemia viruses (MLVs) (Barnard et al., 2006; Kozak, 2015). Treatment with tunicamycin, inhibitor of N-linked glycosylation, has been shown in some cases to abrogate resistance to retrovirus entry, by deglycosylation of the cellular receptors or the virus envelope proteins (Koo et al., 1994; Miller and Miller, 1992). However, tunicamycin treatment did not rescue sensitivity to CrERV upon infection of either deer cells or the chronically infected human cells (data not shown).

There is an intriguing paradox between the xenotropic nature of CrERV and its high efficiency of generation of new germline integrations in mule deer lineage in recent past. One possible solution to this paradox is that endogenous CrERVs are not expressed in the germline cells and therefore do not block the entry receptors. Alternatively, more variants of CrERV may exist that differ in receptor usage and can overcome the interference blocks, a mechanism described in FeLV and KoRV (Overbaugh et al., 2001; Xu et al., 2015). Even more complex mechanism was described in PERVs, where disruption of a highly conserved PHQ motif in the envelope glycoprotein enables transactivation of such viruses by unrelated gammaretroviral envelopes (Lavillette and Kabat, 2004). The PERVs with disrupted PHQ motif gain the ability to infect cells that lack the cognate PERV receptors and also to overcome restrictions caused by receptor interference. This property was suggested to provide novel opportunities to infect germ cells (Lavillette and Kabat, 2004). Interestingly, we observe a tendency toward disruption of the PHQ motif in the evolutionarily young CrERVs (D.E., personal communication). The analysis of the possible underlying mechanisms is under way in our laboratory.

Materials and methods

Cells and co-cultivation protocol

Human rhabdomyosarcoma cell line A-673 (ATCC product number CRL-1598) and primary *O. hemionus* kidney cells (OHK, ATCC product number CRL-6193) were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum, penicillin and streptomycin. HEK 293 T cells were grown in the same conditions, with serum supplements changed to 4% fetal calf serum and 4% calf serum. HEK-293 T cells producing PERV 14/220 (Bartosch et al., 2004) were used as a source of porcine gammaretroviral particles. All cells were cultured in a humidified incubator at 37 °C and 5% CO_2. The co-cultivation experiment was started by mixing equal numbers of deer CRL-6193 and human A-673 cells. Every week, fresh cells from both species were added to the coculture at 1:1 ratio. At indicated time points, samples of the culture fluids were harvested for the RT assay. The samples were spun at 3000 RPM for 5 min to remove cell debris, filtered by a 0.22 μm syringe filter and frozen at −80 °C before further use.

PERT assay

The PERT assay was adapted from previously published protocols (Lovatt et al., 1999; Pizzato et al., 2009; Sears and Khan, 2003). The samples (2 μl of culture supernatant or gradient fraction) were lysed in 8 μL of solution containing 1% TRITON X-100, 0.4 U/μL RNasin (Promega), and 1x ProtoscriptII buffer (New England Biolabs) at room temperature for 30 min. Then, two mastermixes (A and B) were prepared, with the following amounts per one reaction: Mix A contained 20 ng of the template RNA of MS2 phage (Roche), 0.5 ul of the reverse primer (5′- GCCTTAGCAGTGCCCTGTCT) and 10.1 ul water. Mix B contained 3.6 ul of 5x ProtoscriptII buffer, 2 ul of 100 mM DTT, 0.8 ul of 10 mM dNTP2, and 6.4 ul water. Mix A was incubated at 65 °C for 5 min and slowly cooled down to allow primer annealing. Next the mixes A and B were pooled and aliqoted by 18 ul. To each aliquot, 2 ul of the lysates were added and incubated at 37 °C for 30 min (reverse transcription step), then inactivated at 70 °C for 10 min, The newly generated MS2 cDNA was quantified by real-time PCR assay with forward (5′- AACATGCTCGAGGGCCTTA) and reverse primers and probe (FAM-TGGGATGCTCCTACATG-TAMRA). Each reaction contained 1.5 ul of the cDNA sample, 1xqPCR master mix (Eurogentec, Seraing, Belgium), 7.5 pmol of each primer and 3.75 pmol of probe in a total volume of 15 ul. The samples were run on a Bio-Rad CFX96™ Real-Time instrument with a three-step protocol: 1 cycle of 10 min at 95 °C and then 45 cycles consisting of 15 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Cycles of quantification (Cq) values

were generated by the CFX Manager software. With each run, one calibrator sample was assayed and all values were expressed as relative values compared to the calibrator.

Iodixanol gradient

Iodixanol (OptiPrep™) was purchased from Axis Shield (Dundee, United Kingdom). Thirty milliliters of cell free supernatants from virus-producing cells were cleared from cellular debris by low-speed centrifugation (3000 RPM for 5 min at 4 °C) and then centrifuged through a 20% iodixanol cushion in a SW28 rotor (Beckman Coulter Pasadena, CA) for 2 h at 23,000 RPM. The centrifuged pellet was resuspended in 1 ml of PBS. Two-milliliter layers containing 50%, 40%, 30%, 20%, and 10% iodixanol were pipetted in tubes for the SW41Ti rotor (Beckman Coulter) and the resuspended pellet in PBS was applied on top. The gradient was centrifuged for 17 h at 35,000 rpm at 4 °C. Gradient fractions were collected from the top and their density was determined by refractometry. Aliquots from each fraction were used for the PERT assay.

Electron microscopy

Virus particles from the culture medium of infected cells were pelleted by ultracentrifugation as described above and fixed in 2% formaldehyde. Samples negatively stained with 3% phosphotungstic acid (PTA) were then viewed with Jeol JEM, 2000 CX microscope (JEOL, Arishima, Japan).

Plasmid construction

For the preparation of replication-competent CrERV DNA clone, two partially overlapping proviral fragments were separately amplified from infected HEK-293 T cells using PCR. Primers for amplification of 5′ CrERV fragment were: 5′–AACCGCGGCCGCTGTAGGGAGAACAAACGGAATGTAGAAAG-3′ (NotI restriction site used for cloning is underlined) and 5′–CAGGGGTAGGCTGAAAAAGGCATC-3′. Primers for amplification of 3′ CrERV fragment were: 5′–TACCCATATGTGTTATGCCCGATGTCCGAATCC-3′ (NdeI restriction site used for cloning is underlined) and 5′–GCCCTCAGAGGTCATAGCACCAGA-3′. Amplified DNA fragments covered the entire proviral sequence and contained unique EcoRI restriction site in the overlapping region. The DNA fragments were digested with NotI/EcoRI and NdeI/EcoRI for the 5′ and 3′ regions, respectively. Consequently, both cleaved fragments were sequentially ligated into the pGem-T Easy cloning vector (Promega, Madison, WI), creating intact CrERV proviral DNA clone (pCrERV-IND). To create the modified variant of CrERV (CrERV-mut), 298 bp-long region of viral *pol* gene was PCR-amplified using mutagenic primers which contain several mismatching bases and EcoRI/HpaI restriction sites naturally occurring in viral sequence: 5′–CCGGAATTCTTCGTCGGTGCCAA and 5′–CAAGTTAACTGCACCACAGGTTGG. Original sequence in pCrERV-IND plasmid was replaced by this mutated fragment using EcoRI and HpaI enzymes, which generated the pCrERV-mut construct.

Virus preparation and cell infection

HEK-293 T cells were transfected with pCrERV-IND or pCrERV-mut plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche s.r.o., Prague, Czech Republic). Specifically, 5×10^4 cells were seeded onto 24-well plate and next day transfected by addition of 100 μl of culture medium containing 0.5 μg of DNA and 0.5 μl of the transfection reagent. Cells were grown for approximately 30 days until the viral DNA copy number reached plateau. Afterwards, 100 μl of filtered cell supernatant was used for infection of naïve HEK-293 T cells seeded on 24-well plate (5×10^4 per well) a day before. After 1 h of incubation at 37 °C, 400 μl of fresh medium was added, and the cells were further incubated in CO_2 at 37 °C. For determining the infectious titer of CrERV, 10-fold dilutions of virus were inoculated in triplicate wells. The cells were passaged for 4 weeks to allow virus spread, and scored for virus presence using both PCR assays and PERT assay.

PCR-based detection of viral DNA (qPCR, digital droplet PCR and standard analytical PCR)

For the determination of reversely-transcribed viral DNA, infected cells were harvested at appropriate time points, washed by PBS, and incubated in lysis buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.2 mM $CaCl_2$, 0.001% Triton X-100, 0.001% SDS, 1 mg/ml proteinase K) for 60 min at 58 °C, followed by 10 min protease heat-inactivation at 95 °C. Primers for quantitative PCR (5′–TGACCCCATGTTTGAATGTG and 5′–GAGGACAGCTCCTTGGTTTG) were designed to conserved region of CrERV *env* gene using Primer3Plus software (Untergasser et al., 2007). For the quantification of PERV DNA, primers 5′–AGGTGGTGGGCATGTAATACTG and 5′–ACACTCGGGGAACAATTTGG, also situated in *env* gene, were used. MESA GREEN qPCR mastermix (Eurogentec, Seraing, Belgium) was used for standard real-time quantitative PCR. Each reaction mixture had a total volume of 20 μl, containing 2 μl of the cell lysate and 300 nM (each) the forward and reverse primers. The samples were run on a Bio-Rad CFX96™ Real-Time System (Bio-Rad, Hercules, CA) with a two-step protocol (1 cycle of 5 min at 95 °C and then 44 cycles consisting of 15 s at 95 °C and 60 s at 60 °C), followed by melting curve analysis in the CFX Manager software (Bio-Rad) to ensure the specificity of the amplification. An absolute standard curve for each assay was obtained by using as templates serial dilutions of a plasmid containing the corresponding amplicon. The results were normalized using the parallel amplification of a single-copy genomic locus in porphobilinogen deaminase gene (Konig et al., 2008).

For highly accurate absolute quantification of viral DNA, droplet digital PCR system QX200 (Bio-Rad, Hercules, CA) was used. Each reaction mixture had a total volume of 20 μl, containing 1x QX200 ddPCR Evagreen Supermix (Bio-Rad), 2 μl of the cell lysate (1–5 ng DNA), and 250 nM (each) the forward and reverse primers. The reactions were treated for droplet generation according to the manufacturer's manual and then amplified with the following conditions: 1 cycle of 5 min at 95 °C and then 40 cycles consisting of 15 s at 95 °C and 40 s at 59 °C followed by 1 cycle of 5 min at 72 °C, 5 min at 4 °C and 5 min at 90 °C. Samples were analyzed by droplet reader and QuantaSoft software (Bio-Rad) with thresholds set manually.

For specific detection of modified CrERV variant (CrERV-mut) in infected cells, PCR primers complementary to the modified region of viral DNA were designed: 5′–GGATGCCGGAATTCTTCGT and 5′–GTCCAAGTTAACTGCACCACA. Primers complementary to the wild-type CrERV sequence were used as a positive control: 5′–GGATGCCGGAATTCTCAGG-3′ and 5′–GGGTCCAAGTTAACTGTACTACC-3′. OneTaq mastermix (New England Biolabs, Ipswich, MA) was used for analytic PCR amplification. Each reaction mixture had a total volume of 20 μl, containing 2 μl of the cell lysate and 200 nM (each) the forward and reverse primers. The samples were run with a following protocol: 1 cycle of 3 min at 94 °C and then 32 cycles consisting of 20 s at 94 °C, 30 s at 60 °C and 30 s at 68 °C finished by 1 cycle of 5 min at 68 °C and analyzed by agarose gel electrophoresis.

Phylogenetic tree

The 3′ portion of 13 CrERV sequences (approximately 1100 bp) was aligned using Muscle (Edgar, 2004) and the phylogeny was generated with PhyML (Guindon et al., 2010) and the HKY85 model with a gamma distribution.

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